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Thrombopoietin Is Synergistic With Other Hematopoietic Growth Factors and Physiologic Platelet Agonists for Platelet Activation In Vitro

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Thrombopoietin (TPO) is the primary physiologic regulator of platelet production. The effect of TPO on platelet function, both alone and in combination with other hematopoietic growth factors, adenosine diphosphate (ADP), and epinephrine, was investigated using fluorescent-labeled antibodies to the activation-dependent antigen CD62 (P-selectin) and flow cytometry. TPO stimulated CD62 expression on normal human platelets, and this expression was completely inhibited by the soluble extracellular domain of the TPO receptor, MPL. The growth factors granulocyte colony-stimulating factor (G-CSF) and erythropoietin (EPO), but not interleukin-3 (IL-3) or stem-cell factor (SCF), also stimulated platelet activation. The combination of EPO, SCF, ADP, and epinephrine with TPO were synergistic for platelet CD62 expression. These data further support a role for TPO in modulating platelet function. Am. J. Hematol. 54:225–232, 1997

Key words: thrombopoietin; P-selectin; platelet activation; growth factors

INTRODUCTION

Thrombopoietin (TPO), the ligand for the c-mpl receptor, has recently been cloned and characterized by several groups and is the primary regulator of platelet production [1–4]. C-mpl-ligand supports the proliferation and differentiation of megakaryocyte progenitors into large, polyploid, platelet-producing megakaryocytes [5,6]. Thrombopoietin levels are inversely related to platelet count [7], and the administration of this cytokine in vivo drives platelet production in mice [5,6], nonhuman primates [8,9], and humans [10]. The therapeutic potential for thrombopoietin, either alone or combined with other hematopoietic growth factors in ameliorating the thrombocytopenia associated with primary disorders of bone-marrow function or as the result of aggressive cytotoxic therapy, is enormous.

The receptor for thrombopoietin, c-Mpl is predominantly expressed on megakaryocytes, endothelium, and platelets [11,12], although c-Mpl message has been detected by reverse-transcription polymerase chain reaction in normal human fetal liver and brain tissue [13]. Felder et al. [14] have recently shown that mature platelets bind, internalize, and degrade TPO. Miyakawa et al. [15] have shown that incubation of human platelets with thrombopoietin stimulated protein tyrosine phosphorylation of the signal transduction proteins Janus kinase 2 (Jak2) and Shc, suggesting an effect of TPO in mature platelets. Our group and others have demonstrated that TPO augments the activation response of platelets to agonists [16–18] and directly increases platelet fibrinogen binding and adhesion to collagen [19,20].

Using fluorescent-labeled monoclonal antibodies to activation-dependent platelet antigens and flow cytometry, we previously reported that thrombopoietin activated platelets in vitro [21]; this was recently confirmed by others [22]. In the present study, we confirm our previous findings of in vitro TPO-induced platelet acti-

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vation, and show effects at physiologic levels of TPO. In addition, as combinations of TPO and other hematopoietic growth factors (HGF) are likely to be used in patients [23], and as both the granulocyte colony-stimulating factor receptor [24] and the stem-cell receptor [25] are present on mature human platelets, we determined the effect of cytokine combinations on platelet activation. Our results demonstrate that TPO and other hematopoietic growth factors can activate platelets in vitro. In addition, there is synergy between TPO and physiologic agonists as well as other HGF for platelet activation.

MATERIALS AND METHODS

Study Subjects

Normal, healthy volunteers were recruited from the staff of the Sacramento Medical Foundation Blood Center after approval by the Institutional Review Board of the Sacramento Medical Foundation (n = 15, 12 females and 3 males, ages 23–59). All donors gave written informed consent, were nonsmokers, and had not taken any platelet-active drugs for 10 days prior to the study. Five ml of whole blood were obtained in sodium heparin using an evacuated tube. Previous studies have shown minimal ex vivo activation by this collection method [26–28].

TPO and c-Mpl

Recombinant human TPO (TPO) was expressed and purified from BHK cells from a full-length cDNA, and the soluble extracellular domain of c-Mpl (s-Mpl) was prepared as previously described [1,6]. The concentration of TPO was 1,860 ng/ml and was stored at 4°C prior to use. Conditioned BHK medium was used as a negative control and as a diluent for TPO.

Flow Cytometry Studies

The expression of the activation-dependent platelet antigen P-selectin (CD62) was used to measure the effect of in vitro incubation of peripheral blood platelets with TPO and other recombinant hematopoietic growth factors. Within 5 min of obtaining whole blood as above, tubes were centrifuged at 300*g* for 2 min to obtain ''slow-spin'' platelet-rich plasma (PRP). Fifty μl of PRP were incubated for 5 min at room temperature in a total volume of 500 &l with the following: 1) 0.1 M HEPES (negative control, Sigma, St. Louis, MO), 2) conditioned BHK medium (negative control, ZymoGenetics, Seattle, WA), 3) TPO 0.2–100 ng/ml (ZymoGenetics, Seattle, WA), 4) erythropoietin (EPO), 1 mU 2–500 U/ml (AMGEN, Thousand Oaks, CA), 5) 20 μM ADP (positive control, BioData Corporation, Horsham, PA), and 6) 20 μM epinephrine (positive control, BioData Corporation, Horsham, PA). These concentrations of ADP and epinephrine led to submaximal activation in our system. PRP was also incubated with 100 ng/ml concentrations of the following growth factors: granulocyte colony-stimulating factor (G-CSF) (AMGEN, Thousand Oaks, CA), interleukin-3 (IL-3), and stem-cell factor (SCF) (both from Stem Cell Technologies, Inc., Vancouver, British Columbia, Canada), alone or in combination with varying concentrations of TPO. In some experiments, PRP was preincubated with 2 μg/ml s-Mpl prior to incubation with TPO, EPO, G-CSF, ADP, or EPI, based on data that this would likely inhibit any TPO-mediated effect on platelets [29].

After incubation, 50 μl of each test or control platelet mixture were then added to 12 × 75-mm Falcon plastic tubes containing 20 μl of the following in 150 μl of 0.1 M HEPES: 1) mouse IgG_1 (FITC) and mouse IgG (PE) (isotype control), 2) anti-CD61-FITC (clone RUU-PL7F12) and anti-CD62 PE (clone AC1.2) (test), all from Becton Dickinson Immunocytometry Systems (San Jose, CA). After 30 min of incubation in the dark at room temperature, 1 ml of 1% paraformaldehyde (pH 7.2, freshly prepared and filtered through a 0.2-μm filter) was added to each test and control tube. Samples were stored in the dark at 2–8°C and analyzed within 24 hr on a flow cytometer. Cytometer performance was verified using 1-, 2-, and 10-μm calibration beads (Becton Dickinson, San Jose, CA and Flow Cytometry Systems, Research Triangle Park, NC). Ten thousand events were collected in list mode with all light-scatter and fluorescence parameters in logarithmic mode. Platelets were gated on the basis of light scatter and CD61 expression. Activated platelets were defined as the percentage of CD61-positive events coexpressing CD62 (i.e., exhibiting fluorescence greater than the isotype control for CD62-PE).

Statistical Analysis

Examination of the data revealed a non-Gaussian distribution. Therefore, the Friedman repeated measures analysis of variance on ranks was used to compare CD62 expression between serial data for a given subject. The Student-Newman-Keuls multiple comparison method was then used to identify which groups differed. Wilcoxon signed rank and Mann-Whitney tests were used to determine differences between paired and unpaired data, respectively. $P \leqslant 0.05$ was considered statistically significant. Statistical calculations were performed using Statview 4.02 for the Macintosh (Abacus Concepts, Berkeley, CA) or SigmaStat 1.0 for Windows (Jandel Scientific, San Rafael, CA).

RESULTS

TPO Activates Platelets Via Interaction With c-Mpl

Preliminary studies showed no CD62 expression in response to TPO concentrations below 50 ng/ml. How-

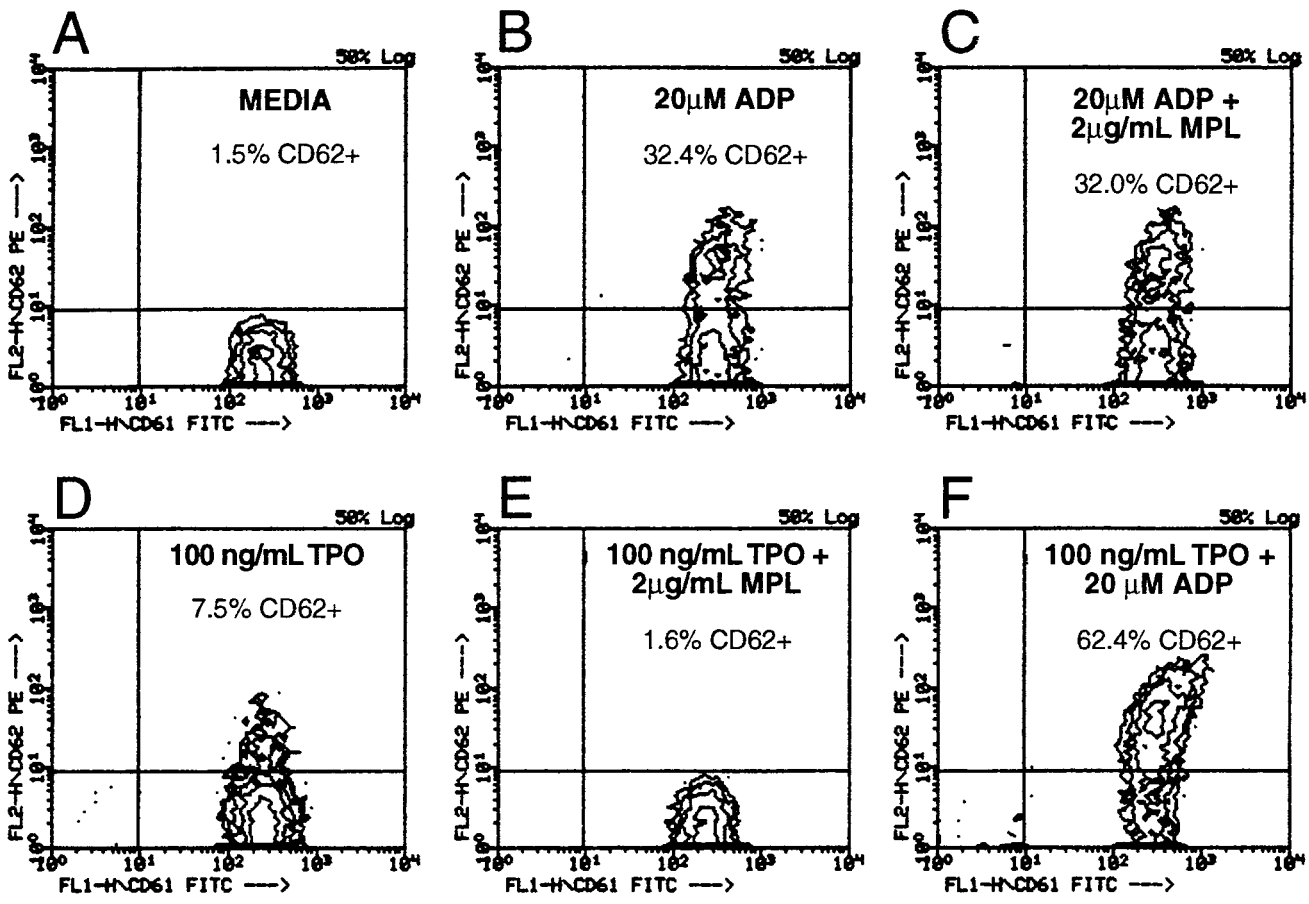

Fig. 1. TPO-induced platelet activation. Contour plots of CD62-expression on platelets in response to the indicated agonists. Events in quadrant 2 (right upper quadrant of each plot) represent activated platelets. Note that there is no inhibition of ADP-induced CD62 expression with s-Mpl, and that TPO and ADP appear to be synergistic for platelet activation.

ever, at a concentration of 100 ng/ml TPO, there was consistent platelet activation (Fig. 1, 2). In order to demonstrate that this CD62 expression was indeed mediated by interaction with c-Mpl, samples were preincubated with s-Mpl before the addition of TPO. As shown in Figure 2, 2 μg/ml s-Mpl abolished TPO-stimulated CD62 expression. Inhibition of TPO-induced platelet activation by s-Mpl might have been due to nonspecific toxicity to the platelets. To determine if this were the case, platelets were also stimulated with ADP after s-Mpl incubation. The median CD62 expression with 20 μM ADP was 46.8 and 50.4 ($P = 0.14$, Wilcoxon signed rank), in the absence and presence of s-Mpl, respectively.

Platelets Are Activated by Other Hematopoietic Growth Factors

Because it had previously been shown that G-CSF receptors [24] and SCF receptors [25] were present on mature human platelets, we examined whether these and other hematopoietic growth factors would stimulate CD62 expression on platelets in vitro. Figure 3 shows consistent platelet activation by G-CSF and EPO, but not

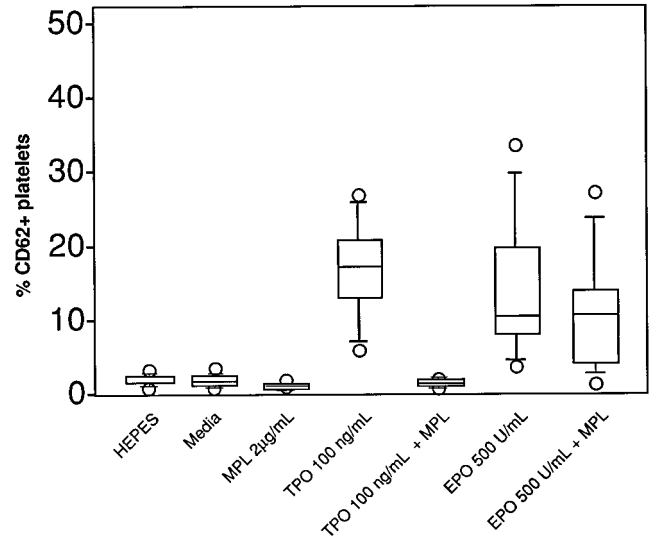

Fig. 2. Inhibition of TPO-induced CD62-expression by s-Mpl. PRP (n = 10) were stimulated with TPO or EPO after preincubation with 2 μg/ml s-MPL. There was significant inhibition of both TPO- and EPO-induced platelet activation (P = 0.005 and 0.007, respectively, Wilcoxon signed ranktest).

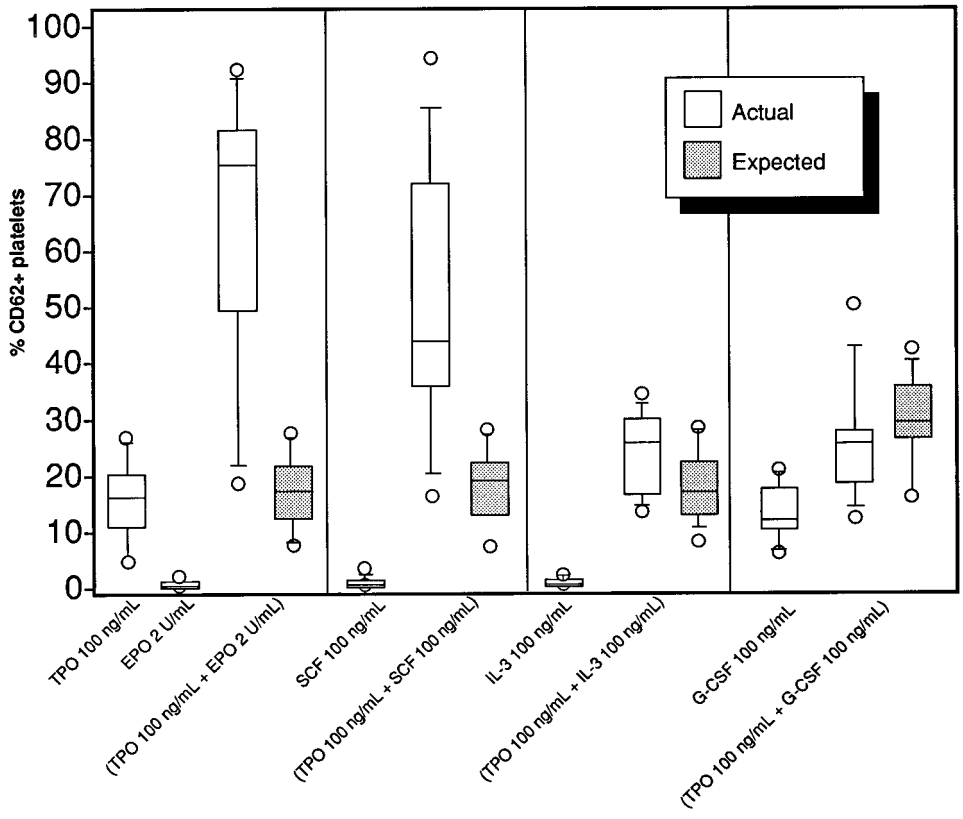

Fig. 3. Synergy between TPO and other hematopoietic growth factors in vitro (n = 15, except G-CSF, n = 10). PRP were incubated with combinations of TPO and other growth factors. Actual percentages of platelets expressing CD62 were compared to expected percentages (see Results) by Wilcoxon signed ranktest. $P \leq 0.05$ for EPO, SCF, and IL-3, but not G-CSF.

by IL-3. Somewhat surprisingly, there was no SCF-stimulated CD62 expression on platelets, as SCF receptors are found on platelets [25].

Synergistic Effect of TPO With Other Cytokines for Platelet Activation

It is likely that combinations of growth factors will be used in the clinic to optimize hematopoietic recoveries of multiple cell lines. In addition, multiple growth factor levels are increased in vivo in the setting of pancytopenia. To detect synergy between TPO and other hematopoietic growth factors, we compared the actual percentage of activated platelets obtained from the combination of TPO and another HGF with the expected value of the percentage of platelets expressing CD62 (expected percentage = percent CD62 expression with TPO alone plus percent CD62 expression with HGF alone). Expected and actual values were compared using the Wilcoxon signed rank test. TPO in combination with SCF, IL-3, and EPO, but not G-CSF, produced greater-than-expected CD62 expression on platelets (Fig. 3).

The physiologic concentrations of TPO in patients with normal platelet counts and those with thrombocytopenia and thrombocytosis have recently been determined [7,30]. In order to determine if physiologic levels of TPO might have an effect on platelets when combined with other hematopoietic growth, platelets were incubated with combinations of TPO and 1 ng/ml each of G-CSF, SCF, and IL-3, as well as 1 mU/ml of EPO. There was no evidence of platelet activation compared to negative controls with any growth factor alone at these concentration. However, the combination of 200 pg/ml TPO and 1 mU/ml of EPO resulted in significant CD62 expression (median 10.7% vs. 5.8% CD 62 expression (expected), $P = 0.04$, Wilcoxon signed rank). In contrast to the results seen at higher levels of growth factors, SCF and IL-3 did not synergize with TPO for platelet activation.

Synergistic Effects of TPO and Physiologic Agonists

In order to determine if TPO was synergistic with the physiologic agonists ADP and epinephrine for platelet activation, increasing concentrations of TPO were incubated with ADP (Fig. 1) and epinephrine. The percentage of platelets expressing CD62 was determined and compared with the expected percentage of platelets expressing CD62 (as defined above) using the Wilcoxon signed

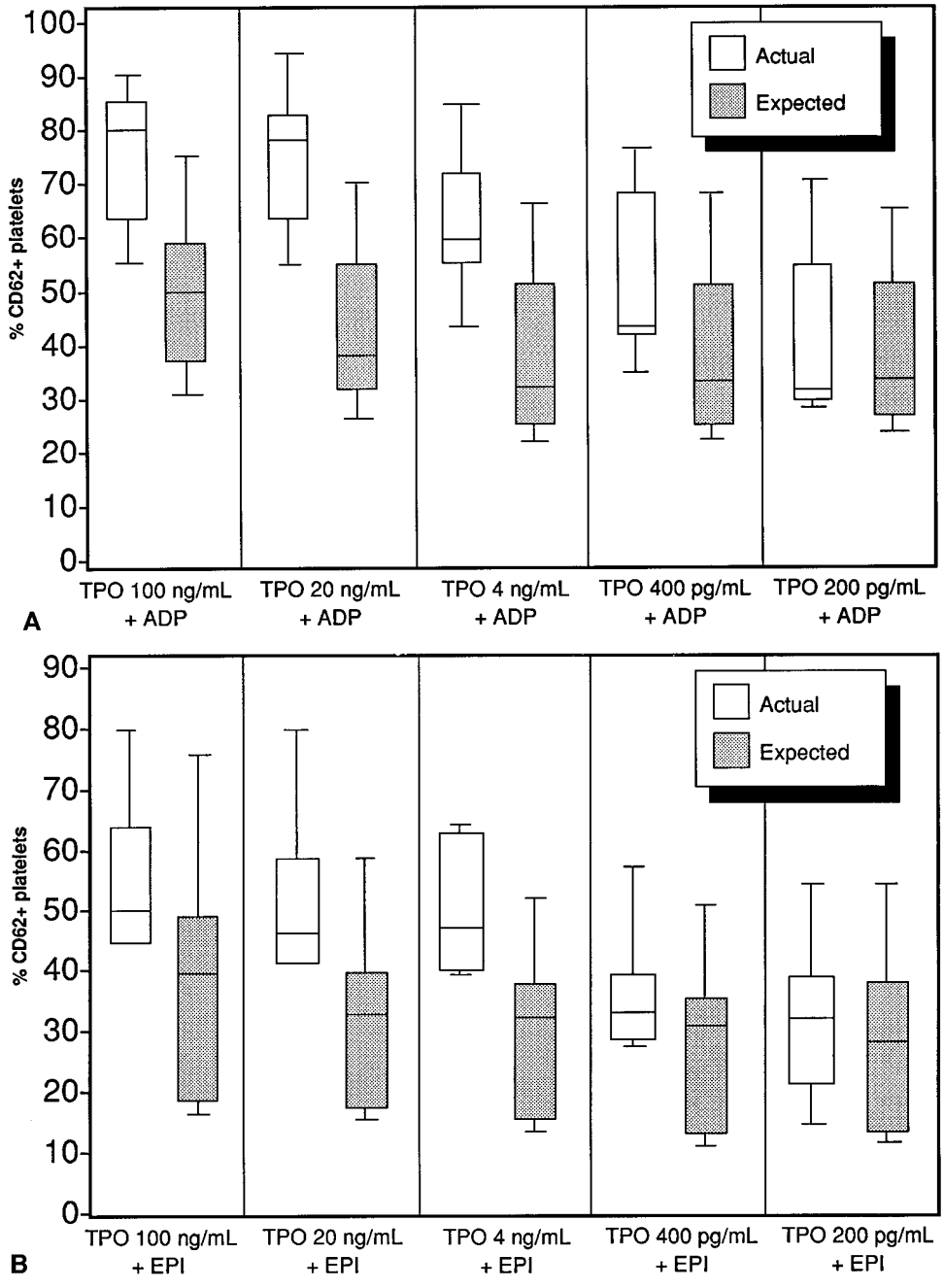

Fig. 4. Synergistic effects of TPO on ADP-stimulated (A) and EPI-stimulated (B) CD62 expression on platelets in vitro. PRP (n = 5) were incubated with varying concentrations of TPO and 20 μM ADP or EPI. Actual percentages of platelets expressing CD62 were compared to expected percentages (see Results) by Wilcoxon signed rank test. $P \leqslant 0.05$ for all comparisons except TPO 200 pg/ml + ADP.

rank test. As can be seen in Figure 4, TPO concentrations of 400 pg/ml or greater produced a greater-than-expected percent expression when combined with ADP and EPI.

DISCUSSION

Thrombopoietin appears to the primary physiologic regulator of megakaryocytopoiesis and platelet production [31]. The results of the present study confirm our previous findings [16,21] and those of others [15,17–20,22] that TPO has a direct stimulatory effect on mature human platelets. Furthermore, platelet activation is mediated by interaction with c-Mpl, as the addition of s-Mpl prior to the addition of TPO abolished the activation of platelets. Analogous to previous studies which demonstrated that the hematopoietic growth factors GM-CSF,

G-CSF, and SCF have effects on mature blood cells [32–34], it appears that TPO acts on mature platelets to increase functional activity, as well as having proliferation and differentiating effects on precursor cells.

The range of the percentage of platelets activated by 100 ng/ml of TPO was 6.7–26.5%. We are unaware of any data to suggest heterogeneity of TPO receptor expression on platelets either within or between individuals that might explain this finding. However, we previously demonstrated that the range of activation-marker response to ADP is highly variable between individuals (but very consistent), as is the aggregation response to other weak agonists. These data could also result from the relative insensitivity of our system to minimal platelet effects.

P-selectin (CD62) is a platelet α-granule membrane component that is externalized onto the platelet membrane upon secretion and thus serves as a marker for activation [35,36]. Platelets so activated would be more likely to adhere to sites of vascular injury. Additionally, P-selectin serves as an adhesion molecule for platelet-leukocyte interactions [37,38]. Recruitment of inflammatory cells would further augment the hemostatic cascade.

Erythropoietin receptors are not known to be present on human platelets, though they have been found to be expressed on rodent megakaryocytes [39]. The N-terminal region of TPO has significant sequence homology with EPO (50% homology when accounting for conserved sequences) [8], and thus we hypothesized that the EPO-induced platelet activation seen in this study was the result of interaction with c-Mpl. Preincubation with s-Mpl at a concentration that completely inhibited TPO-induced platelet activation did result in a statistically significant decrease in EPO-induced activation ($P = 0.007$, Wilcoxon signed rank), though not to the degree that it inhibited TPO-induced CD62 expression (Fig. 2) despite similar degrees of activation at the concentrations studied. In addition, there was no correlation between EPO- and TPO-induced platelet activation. ($\rho = 0.375$, $P = 0.16$, Spearman rank correlation). Thus, we are unable to draw conclusions about the mechanism of EPO-induced platelet activation. Studies using radiolabeled TPO and EPO that are underway will answer questions regarding their interaction with c-Mpl (Kaushansky, unpublished data). It should be noted that the levels necessary to demonstrate a platelet-activation effect of EPO are suprapharmacologic by nearly two orders of magnitude [40].

Shimoda et al. [29] previously demonstrated the presence of G-CSF receptors on mature human platelets using flow cytometry and radioreceptor assay studies. Using platelet aggregometry as measured by light transmittance, these investigators were unable to demonstrate a direct effect of G-CSF on platelet aggregation. However, a concentration G-CSF as low as 0.1 ng/ml was able to augment the aggregation response to submaximal concentrations of ADP, and there was a concentration-response relationship up to 10 ng/ml. We were able to demonstrate a direct stimulatory effect of G-CSF on platelets, but only at concentrations above the physiologic range (which can range from undetectable to close to 4,000 pg/ml with bacterial infections) [41,42]. However, serum levels of G-CSF can reach upwards of 100 ng/ml after subcutaneous administration [43–45], and thus platelet activation and subsequent platelet clearance could account for the moderate thrombocytopenia seen with large doses of G-CSF [46].

In agreement with the report by Grabarek et al. [25] and our previous work [16], we were unable to detect a platelet activation effect of 100 ng/ml of SCF in vitro. These previous investigators were able to demonstrate augmentation of the second wave of platelet aggregation in response to ADP or epinephrine with concentrations of SCF of 50–200 ng/ml, but these concentrations are higher than physiologic levels of SCF [47–50]. Levels of IL-3 can reach up to 6,000 pg/ml after myeloablative therapy [51], and 135 ng/ml after intravenous bolus [52]. We did not observe any effect of 100 ng/ml IL-3 on platelet activation, and we are not aware of any data showing the presence of IL-3 receptors on platelets.

Thrombopoietin augments the activation response of platelets to the physiologic agonists ADP and epinephrine, in agreement with previous data with platelet aggregation [15–18,21]. These data further support a role for TPO in physiologic platelet activation [19].

Thrombopoietin may be combined with other cyotkines in order to maximize thrombopoiesis and stimulate multiple cell lines. Indeed, at least in vitro, combinations of SCF and TPO [53] and EPO and TPO [53] act synergistically for colony-forming unit-megakaryocyte numbers, and the combination of IL-3 and TPO are at least additive [53]. There was clear synergy of TPO and EPO, and TPO and SCF (Fig. 3). Erythropoietin synergy with TPO was seen at pharmacologic and physiologic levels of EPO, whereas the effect with SCF was only seen at supraphysiologic levels (which is around 2 ng/ml) [48,49]. Hematopoietic growth factors increased in situations of anemia, and thrombocytopenia could synergistically augment platelet function.

In summary, these data demonstrate a direct stimulatory effect of TPO on platelets in vitro. In addition, TPO combined with EPO, SCF, or IL-3 appears to be synergistic for platelet activation. This is not the case with G-CSF. SCF, IL-3 and G-CSF are hematopoietic growth factors that may be useful in conjunction with TPO to hasten multilineage hematopoietic recovery after aggressive cytotoxic therapy. The clinical implications for these findings are uncertain; however, animal studies to date have not revealed an increased thrombotic tendency with

TPO [54]. Indeed, in thrombocytopenic patients, augmentation of platelet function may be a desirable effect.

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